Functional Analysis of Haplotypes and Promoter Activity at the 5' Region of the ADH7 Gene

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Abstract

Background: The function of the 5' regulatory region and the role of the different SNP loci have not been well characterized. This study investigated the effect of several ADH7 haplotypes on the regulation of gene expression in vitro and the functional sequences in the 5' regulatory region of ADH7. Three SNPs (rs17537595, rs2851028, and rs2654847) and four different haplotypes (T-C-T, T-T-T, C-T-T, T-C-A) were identified by cloning and sequencing.

Methods: Effects of 4 different haplotypes and 8 truncated fragments of 5' regulatory region on *ADH7* gene expression were detected using a dual-luciferase reporter assay system. All recombinant plasmids were transfected into HEK-293, U87, and SH-SY5Y cells, respectively, and their relative fluorescence intensity was measured.

Results: In HEK-293, U87, and SH-SY5Y cell lines, the relative fluorescence intensity of haplotype T-C-T was significantly higher than that of haplotype T-T-T, C-T-T, and T-C-A. Additionally, we found that regions from -83 to -310bp (ATG, +1), -560 to -768bp, and -987bp to -1203bp up-regulated gene expression. In contrast, the region from -768 to -987bp down-regulated gene expression. The gene expression of regions from -1203 to -1369bp and -1369 to -1626bp was down-regulated in U87 and SH-SY5Y cell lines, but the trend was opposite in HEK-293 cell line. The region from -310 to -560bp up-regulated gene expression in SH-SY5Y cell line, but down-regulated gene expression in HEK-293 and U87 cell lines.

Conclusions: This study has shown that the polymorphisms of ADH7 5' regulatory region play an important role in the regulation of gene expression.

1. Introduction

The *ADH7* gene encodes IV alcohol dehydrogenase, which is mainly expressed in the upper digestive tract. ADH7 mostly plays a role as retinol dehydrogenase to convert retinol (the main precursor of vitamin A) to retinal, relates to the synthesise of retinoic acid, and regulates the metabolic pathway and metabolic process of retinoic acid(Ding et al. 2020). Extensive genomic studies have confirmed the association between the *ADH7* gene and diseases such as alcohol dependence, drug dependence, severe depression, Parkinson's disease, and upper gastrointestinal cancer(Buervenich et al. 2000; Luo et al. 2008; Wang et al. 2014; Zuo et al. 2013).

Gene expression is typically regulated at the transcriptional level through the binding of transcription factors to genomic regulatory regions called promoters and enhancers(Wang et al. 2018). The genomic regions with protein-coding capacity only account for 1% of the human genome and a vast proportion of non-coding genomes are more likely correlated with the genetic associations of many diseases(Hindorff et al. 2009). Sowmya Jairam *et al.* have shown that ADH7 proximal promoter variant rs2851028 affect transcriptional activities. Upstream regulatory sequences generally showed a greater increase or smaller reduction in activity when combined with the rs2851028-T promoter than with the rs2851028-C promoter(Jairam and Edenberg 2014). A previous study showed that the binding of transcription factors

AP-1 and C/EBP to the promoter region of the *ADH7* gene significantly reduced its activity(Kotagiri and Edenberg 1998).

We have identified three SNPs in the 5' proximal promoter region of the *ADH7* gene previously, but the influence of haplotypes and different fragments of the proximal promoter region on the protein in vitro is still not clear(Zeng et al. 2020). Therefore, we further explored the effects of four haplotypes and multiple recombinant fragments on gene expression at the protein level.

2. Materials And Methods

2.1. Ethical compliance

The study was approved by the China Medical University Review Committee (Institutional Review Board number: [2016]063). All samples were collected following the principle of informed consent.

2.2. Construction of recombinant vectors of four haplotypes in the 5' promoter region of *ADH7* gene

According to our previous research(Zeng et al. 2020), DNA samples containing four haplotypes (T-C-T, T-T-T, C-T-T, and T-C-A) were selected as templates to amplify target fragments by PCR.

Primers were designed and restriction enzymes were selected according to the pGL3 plasmid polyclonal site using Primer 5.0 software. The restriction endonucleases *Kpn*I and *Hind*III were introduced into the 5' end of the corresponding primers, and the target fragment was amplified by PrimerSTAR® reagent kit (Takara, Dalian, China). The primer sequences were 5'-CGGGGTACCGATTTGGAGTCAGATG-3' (sense) and 5'-CCCAAGCTTCATCCTGTCTTTGTCTTG - 3' (antisense). The underlined nucleotides were used to introduce restriction sites for *Kpn*I or *Hind*III. The purified PCR product was cloned into pBM20S vector by pBM20S Toposmart cloning kit (TIANGEN, Beijing, China). The recombinant pBM20S vector was transformed into JM109 competent cells, and the plasmid extracted in the SanPrep® Column Endotoxin-Free Plasmid Mini-Preps Kit(Sangon Biotech, Shanghai, China) was sequenced to confirm that the inserted fragment was correct.

Finally, four haplotypes of pBM20S vectors in the 5' proximal promoter region were subcloned into pGL3 basic vector (Promega, Madison, Wisconsin, USA) and transfected into eukaryotic cell lines.

2.3. Construction of truncated sequence recombinant vectors in the 5' promoter region of *ADH7* gene

The primers (Table 1) containing restriction endonucleases *Kpn*I and *Bgl*II digestion sites were introduced into the 5' end to amplify the target fragment. In this study, the common end was at + 107bp (ATG, + 1) and the longest fragment extended from - 1626bp to + 107bp (ATG, + 1). The longest fragment was used as an amplification template to construct eight truncation fragments with 5' deletion. The purified target gene was cloned into pBM20S vector, and the recombinant vector of the target fragment was accurately

screened by sequencing. Then the pBM20S recombinant vectors were cloned into pGL3 Basic vector for transfection of eukaryotic cell lines.

Table 1
Primer sequences of the target fragments containing the cleavage sites

Target fragments	Primer sequences
<i>KpnI</i> (-1626)F	5'-GGGGTACCCAGATATTAAGCCT-3'
<i>KpnI</i> (-1369)F	5'-GGGGTACCTTCTTCAGCACTAC-3'
<i>KpnI</i> (-1203)F	5'-GGGGTACCGTTATATTGAAAGAG-3'
<i>KpnI</i> (-987)F	5'-GGGGTACCTTTCTTGGAGGT-3'
<i>KpnI</i> (-768)F	5'-GGGGTACCCTCTGGCATTTC-3'
<i>KpnI</i> (-560)F	5'-GGGGTACCTCATCCATATTGATAT-3'
<i>KpnI</i> (-310)F	5'-GGGGTACCGAAGTAACCTAAC-3'
<i>KpnI</i> (-83)F	5'-GGGGTACCGCCAGACTATCT-3'
<i>BglI</i> (+ 107)R	5'-GAAGATCTTCATCATTAGGTCATGAT-3'
Notes: The primer sequences were shown, and the 5' end of the primer sequences contained the <i>KpnI</i> and <i>BglI</i> restriction enzyme cleavage sites. The number in brackets was the 5' end position in the <i>ADH7</i> gene as ATG + 1. F = forward, R = reverse.	

2.4. Cell culture and transfection

Embryonic kidney (HEK-293), glioma (U87), and human neuroblastoma (SH-SY5Y) cell lines were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai) and cultured in a humidified 37 °C environment at 5% CO₂. HEK-293 cells and U87 cells were cultured in HyClone® DMEM high glucose medium containing 10% fetal bovine serum (Thermo Fisher Scientific, Massachusetts, USA), while SH-SY5Y cells were cultured in HyClone® DMEM/F-12 mixed medium containing 15% fetal bovine serum. When the cell density reaches 90% or higher, the transfection experiment was carried out. The cells were inoculated on 24-well plates with 2 × 10⁵ cells per well. According to the manufacturer's agreement (Invitrogen, California, USA), the pGL3 recombinant plasmid containing 4 haplotypes and 8 truncated fragments was co-transfected with Lipofectamine® 3000 reagent and Renilla luciferase expression vector pRL-TK (Promega). After 24 hours of culture, the cells were collected. Finally, firefly luciferase activity (LUC) and renal cell luciferase activity (TK) were measured. In each experiment, recombinant vectors were represented in triplicate and the experiment was repeated three times.

2.5. Statistical analysis

LUC/TK was calculated as the relative fluorescence intensity. The comparison between the different recombinants was performed using SPSS19.0 software (IBM, Armonk, NY, USA). The relative fluorescence

intensity was expressed by mean $\pm$ SD (standard deviation) and $p < 0.05$ (bilateral) represents a statistically significant difference.

2.6. Transcription factor prediction

The functional fragments with important protein expression were analyzed by bioinformatics. Transcription factor prediction for the functional fragments was carried out using JASPAR (<http://jaspar.genereg.net>) (Fornes et al. 2020). The transcription factor with at least > 80% match score was screened.

3. Results

3.1. Construction of luciferase reporter gene vectors in the 5' proximal promoter region of *ADH7* gene

The *ADH7* gene fragment from - 649bp to + 46bp (ATG, + 1) contained three SNPs (Fig. 1). Haplotypes in the 5' proximal promoter region were identified by cloning and sequencing. These sequences included four different haplotypes (5 - 1, 2, 3, 4) (Table 2). In the previous study, we found that haplotype 5 - 1 was the major haplotype in the northern Han Chinese population. Moreover, eight *ADH7* gene sequences of different lengths were successfully cloned into the pGL3 Basic vector (Fig. 2). After sequencing, the inserted fragment sequences were consistent with the primer design fragment sequences.

Table 2
The polymorphism comparison of four SNPs in the haplotype recombinants

Haplotype	rs2654847	rs2851028	rs17537595
5 - 1	T	C	T
5 - 2	T	T	T
5 - 3	T	T	C
5 - 4	A	C	T

3.2. Analysis of relative fluorescence intensity of haplotypes in three cell lines

The luciferase reporter gene assay was carried out in three cell lines HEK-293, U87, and SH-SY5Y respectively, which further proved that the 3 SNPs in the 5' proximal promoter region of the *ADH7* gene were involved in the expression of the *ADH7* receptor. In HEK-293, U87, and SH-SY5Y cell lines, the relative fluorescence intensity of haplotype 5 - 1 (T-C-T) was significantly higher than that of haplotype 5 - 2 (T-T-T), 5 - 3 (C-T-T), and 5 - 4 (T-C-A). In HEK293 cell line, the mean LUC/TK of haplotype 5 - 2 was the lowest compared with the other three haplotypes. There are significant differences between type 5 - 1 and type 5

- 2, and type 5 - 2 and type 5 - 3. In the U87 cell line, the mean value of haplotype 5 - 1 LUC/TK was the highest, which was significantly different from other haplotypes. Haplotype 5 - 2 was significantly higher than haplotype 5 - 3 and 5 - 4. In the SH-SY5Y cell line, haplotype 5 - 2 and 5 - 4 was significantly lower than haplotype 5 - 1. In terms of relative fluorescence intensity, the overall luciferase activity in HEK293 cells was relatively higher (Fig. 3).

3.3. Pairwise comparative analysis of relative fluorescence intensity of eight recombinant vectors in three cell lines

In this experiment, 8 recombinant vectors with different fragment lengths were transfected into three cell lines to verify the influence of the regulatory regions of the *ADH7* gene on the expression of ADH7 protein. We found that regions from - 83 to -310bp (ATG, + 1), -560 to -768bp, and - 987bp to -1203bp up-regulated gene expression. In contrast, region - 768 to -987bp down-regulated gene expression. The gene expression of regions from - 1203 to -1369bp and - 1369 to -1626bp was down-regulated in U87 and SH-SY5Y cell lines, but the trend was opposite in HEK-293 cell line. The region from - 310 to -560bp up-regulated gene expression in SH-SY5Y cell line, but down-regulated gene expression in HEK-293 and U87 cell lines (Fig. 4).

4. Discussion

In order to elucidate the characteristics of *ADH7* gene promoter, we confirmed four haplotypes and eight fragments with different sequence lengths by cloning and sequencing the constructed vectors.

The haplotypes 5 - 1 had a significant statistical difference with 5 - 2. A possible reason was the different the base structure of the two haplotypes of the rs2851028 SNP. We also found that the C allele luciferase expression of rs2851028 was significantly higher than the T allele. What's more, our results were consistent with the previous study, in which the promoter activity of the C allele in CP-A and HepG2 cell lines was 1.6 to 2 times that of the T allele(Jairam and Edenberg 2014). The luciferase expression of A allele of rs17537595 was significantly lower than that of T allele in haplotype 5 - 1 compared with haplotype 5 - 4. Therefore, we speculated that the A allele of rs17537595 may inhibit transcriptional activity. Transcription factors are an important group of proteins that regulate gene expression at the transcriptional level by binding to specific DNA sequences. Through JASPAR analysis of functional regions, we found transcription factors that bound to rs2851028, such as vitamin D receptor (VDR). We also found that the transcription factor that bound to rs17537595 was VDR. Therefore, it was reasonable that VDR could inhibit the transcriptional activity of *ADH7* gene by binding to the A allele of rs2851028 and rs17537595. VDR is a member of the nuclear receptor family and it controls transcriptional responses and regulates micro-RNA directed post-transcriptional mechanisms for initiation of an effective immune response. It has been implicated in the regulation of several vital cellular processes, including apoptosis, cell migration, and calcium homeostasis(Costa et al. 2019; Matana et al. 2018). In the U87 cell line, the relative fluorescence intensity of haplotype 5 - 2 was significantly higher than haplotype 5 - 3, which might be due to the increased specific expression of rs2654847 T allele in human

glioma cell lines. As a result, the specific regulatory role of gene polymorphism is complex and depends on the DNA sequence and the cell type.

The promoters of eukaryotes contain various sites with different affinity for the binding of diverse transcription factors. Transcription factors bind to specific DNA sequences in the promoter regions of genes to activate or repress transcription of multiple target genes (Echigoya et al. 2020; Kim and O'Shea 2008). We performed additional detailed truncation analysis of the promoter region in HEK-293, U87, and SH-SY5Y cell lines. We found that regions from – 83 to –310bp (ATG, + 1), –560 to –768bp, and – 987bp to –1203bp up-regulated gene expression. In contrast, region – 768 to –987bp down-regulated gene expression. There may be an enhanced region in the former sequence and an inhibitory regulatory factor in the latter sequence. Through sequence analysis using JASPAR software, many transcription factors, such as ZNF384, ZEB1, and VSX1/X2, were identified in the region from – 83 to –310bp (ATG, + 1), –560 to –768bp, and – 987bp to –1203bp. ZEB1 is at the center of the signal pathway connecting environmental risk factors hypoxia and blood-brain barrier destruction and has become a target related to the pathogenesis of schizophrenia (Leduc-Galindo et al. 2019; Najjar et al. 2017). The – 768~–987bp region also predicted some transcription factors, such as YY1, VDR, and ZNF354C. ZNF354C acts as an inhibitory transcription factor in its *KRAB* domain dependent on specific amino acids. Overexpression of ZNF354C leads to transcriptional inhibition of many genes (Oo et al. 2020). The expression among the three cell lines was not the same in the regions of –310 ~ –560bp, –1203~–1369bp, and –1369~–1626bp. This heterogeneity might be due to the differences in cell type.

In this study, uncertainty remains regarding the regulatory factors and functional elements that actually play a role in each region. Although transcriptional factors have been predicted in some regions, further experiments need to verify whether these transcription factors affect protein expression. Moreover, only the luciferase protein, and not the mRNA, was detected during the final phase of the study, making it difficult to ensure whether the level of mRNA was consistent with protein expression.

5. Conclusions

ADH7 5' regulatory region polymorphisms have regulatory effects on gene expression. Regions – 83 to –310bp (ATG, + 1), –560 to –768bp, –768~–987bp, and – 987bp to –1203bp contain functional sequences that can promote or suppress *ADH7* gene expression. Therefore, it is necessary to carry out further experiments to explore the structure and function of *ADH7* gene.

Declarations

Funding Information

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Data Availability

All data generated and analyzed during this study have been included in this published article.

Authors' Contributions

K.Z conducted the experimental operation, data summary and the preparation of this article. K.Z, Y.L, M.G, Y.P.L, X.X and F.L.X researched the study program. B.J.W and J.Y gave the experimental design ideas and experimental guidance. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and Informed Consent

The experiments complied with the national guidelines and were approved by the Ethics Committee of China Medical University.

Consent to Participate

Each of the subjects or their relatives signed a written informed consent form before participating in this study.

Consent for publication

Not applicable.

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Figures

Figure 1

The cloned fragment spans from -649 to +46 in the ADH7 gene, including 3 SNPs.

Figure 2

Eight pGL3 recombinant vectors containing different fragments. Notes: The figure shows the location of the target fragment in the eight recombinant vectors obtained from the 5' end of the ADH7 gene regulatory region. The longest target fragment is 1733bp from -1626bp to +107bp (A TG is +1). The 3' ends of other amplified fragments were unchanged.

Figure 3

Relative fluorescence intensity of four different haplotypes in HEK-293, U87 and SH-SY5Y cells. Notes: Plasmids 5-1, 5-2, 5-3 and 5-4 were compared with each other. The relative fluorescence intensity is expressed as mean $\pm$ SD; The difference of relative fluorescence intensity was determined by LSD-T test. * ** p < 0.001; * * p < 0.01; * p < 0.05.

Figure 4

Relative fluorescence intensity of eight recombinant vectors in HEK-293, U87 and SH-SY5Y cells. Notes: Comparison was made between adjacent plasmids; The relative fluorescence intensity was expressed as mean $\pm$ SD; The difference of relative fluorescence intensity was determined by LSD-T test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.